

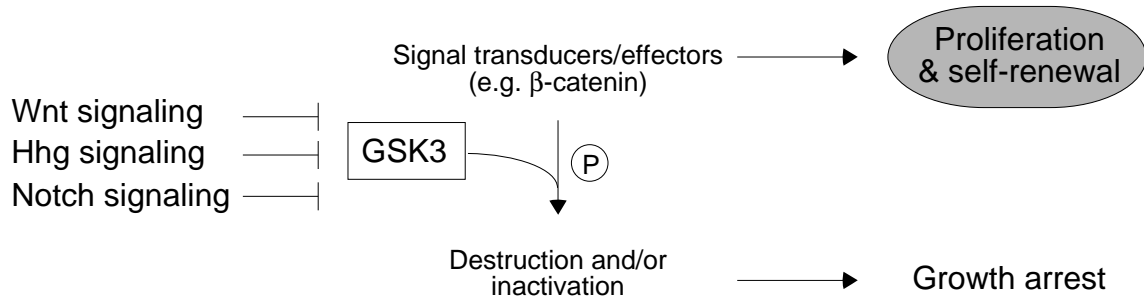
Supplementary Information

The role of glycogen synthase kinase 3 in *MLL* leukaemia maintenance and targeted therapy

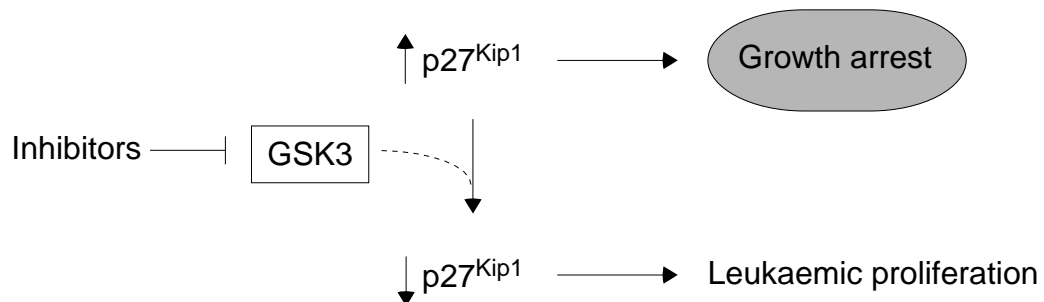
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Normal progenitors

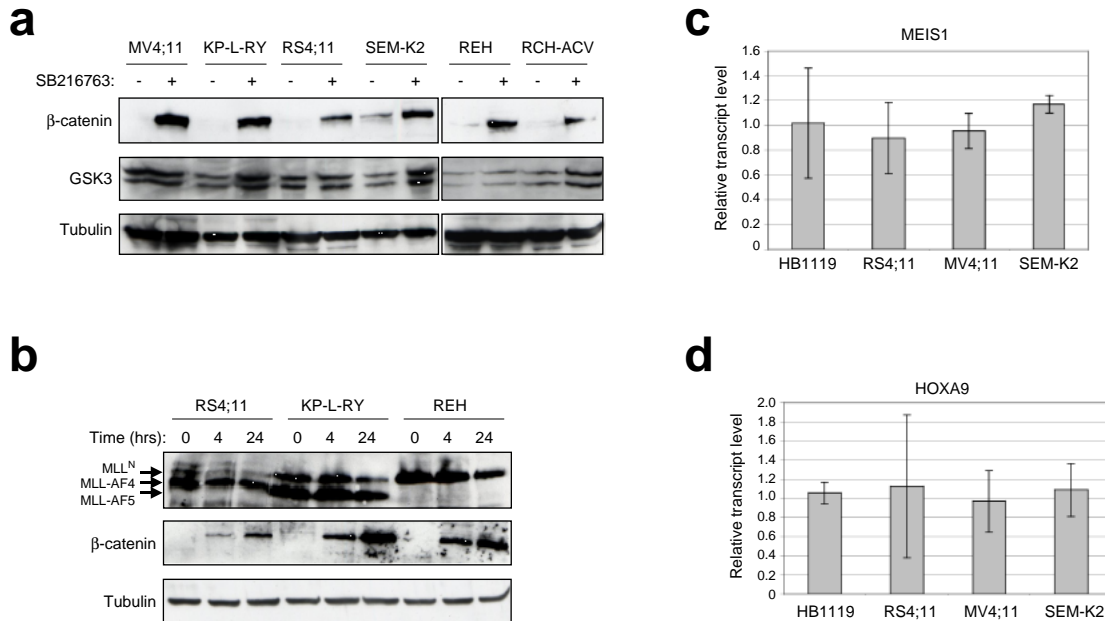


MLL leukaemia



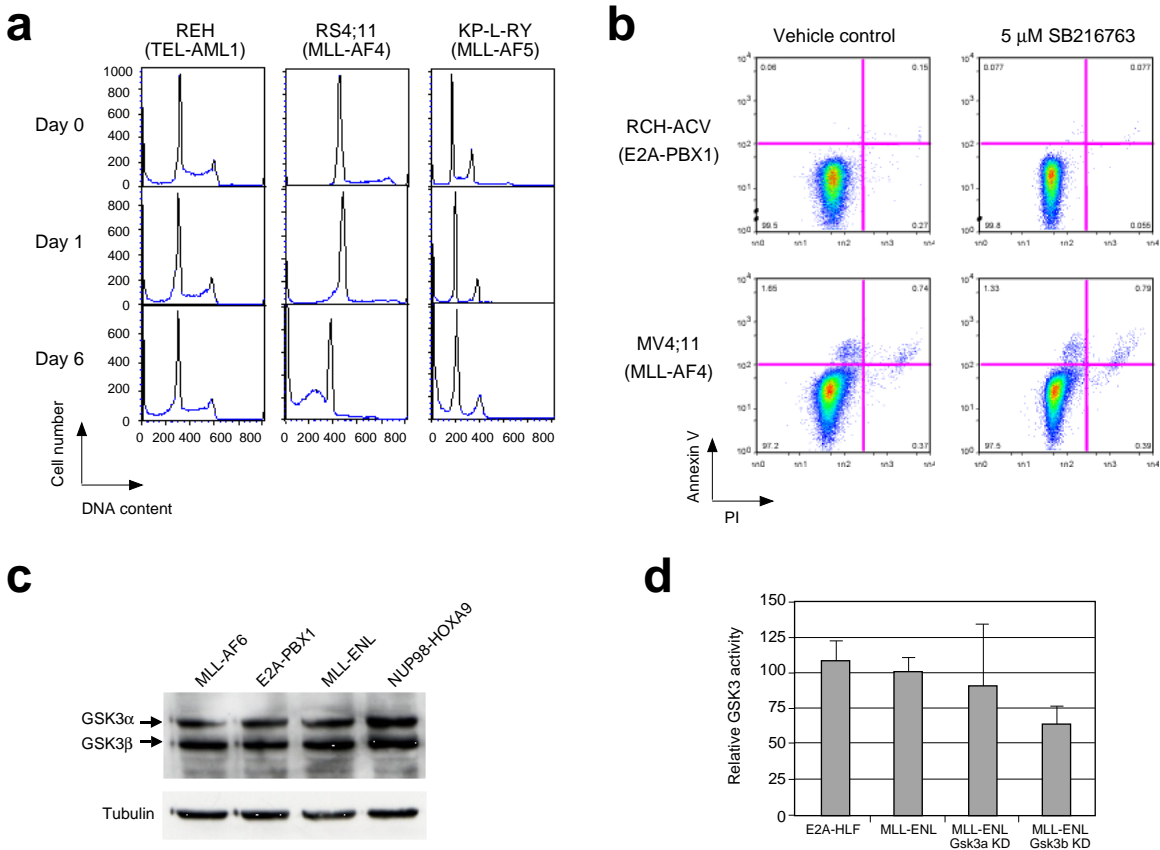
Supplementary Figure 1. Converse roles for GSK3 in normal progenitors versus *MLL* leukaemias.

In normal progenitors, GSK3 mediated phosphorylation results in destruction or inactivation of downstream signaling proteins such as β -catenin. Wnt, Hhg or Notch signaling inhibits GSK3 activity resulting in stabilization of its substrates, and subsequent activation of subordinate gene expression programs that orchestrate progenitor proliferation and self-renewal. This normal suppressive role of GSK3 is circumvented in some cancers leading to inappropriate activation of the Wnt pathway. Conversely, GSK3 activity paradoxically sustains *MLL* leukaemias by reducing p27 levels through unknown mechanisms allowing for unrestrained leukaemic proliferation that is antagonized by GSK3 inhibitors. The disparate outcomes resulting from GSK3 inhibition in normal progenitors versus *MLL* leukaemia cells (indicated with shaded text) may provide significant therapeutic selectivity.



Supplementary Figure 2. GSK3 inhibition does not alter MLL oncoprotein abundance or function.

a, Human leukaemia cell lines (indicated above) were cultured in the absence (-) or presence (+) of SB216763 (10 μ M) for 24 hours, and then analyzed for protein abundance (indicated on left) by western blot. **b**, Human leukaemia cell lines were cultured in SB216763 for the indicated times (top) and then analyzed by western blot analysis for the indicated proteins (left). **c**, **d**, Quantitative RT-PCR was performed on RNA isolated from human leukaemia cell lines cultured in the presence or absence of SB216763 for 24 hours. Relative change in transcript levels (plus inhibitor/minus inhibitor) are shown for *MEIS1* (**c**) and *HOXA9* (**d**) (\pm s.e.m., $n = 3$).

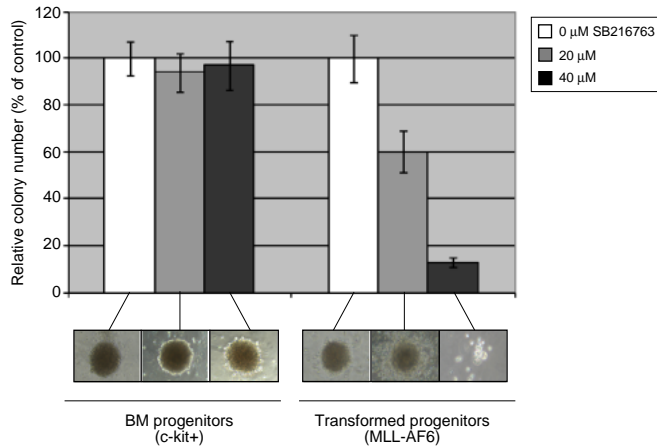


Supplementary Figure 3. GSK3 inhibition specifically antagonizes cell cycle progression of *MLL* transformed cells.

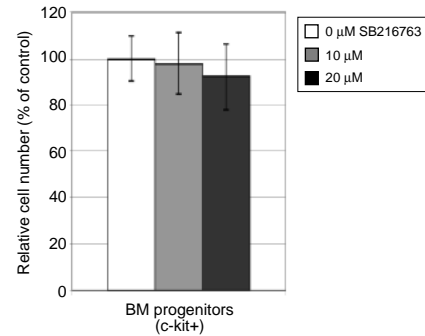
a, Human leukaemia cell lines were incubated with 10 μ M SB216763 for the indicated times and then DNA content was quantified by FACS analysis using PI staining. **b**, Human leukaemia cell lines were incubated with 5 μ M SB216763 for 24 hours at which point apoptosis assays were performed as described in the METHODS. **c**, GSK3 protein levels in myeloid progenitors transduced by fusion oncogenes (indicated at the top) were determined by western blot analysis. **d**, Myeloid progenitors transduced with fusion oncogenes (indicated below) with or without *Gsk3* knockdown were analyzed for GSK3 activity. Results are expressed relative to *MLL-ENL*-transformed wild type cells (\pm s.e.m., $n = 3$).

a

Colony forming unit assays in methylcellulose culture

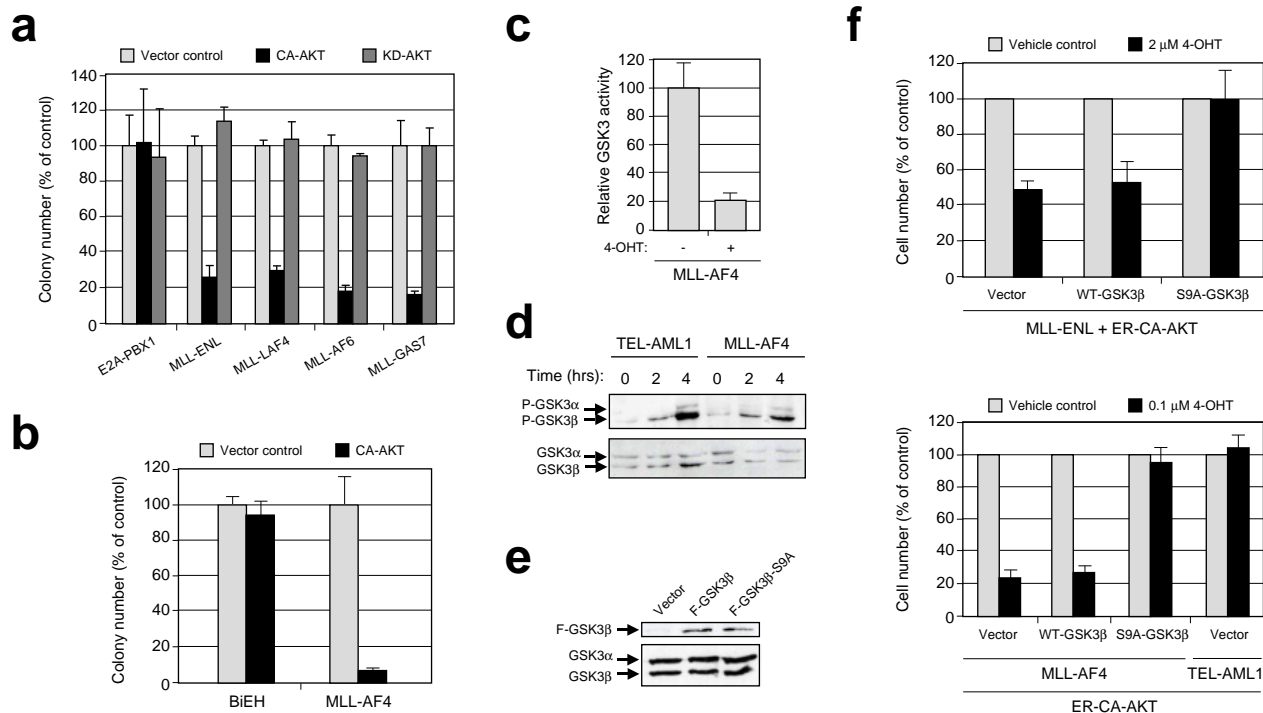
**b**

Growth in liquid culture



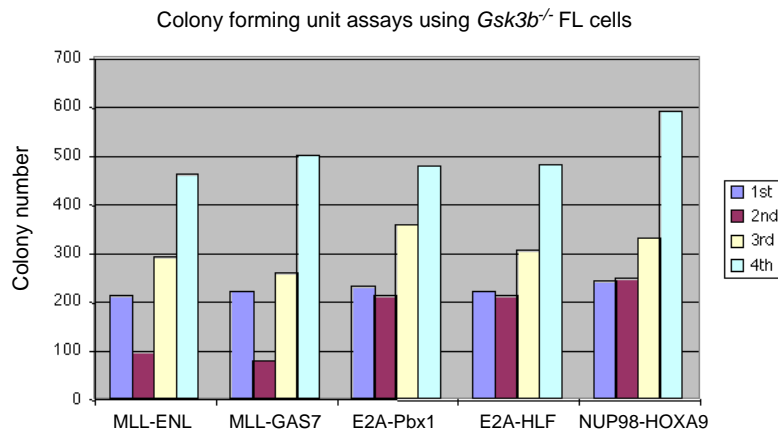
Supplementary Figure 4. Normal bone marrow progenitors are resistant to the adverse effects of GSK3 inhibition compared to *MLL*-transformed progenitors.

a, b, Bone marrow progenitors (c-kit⁺) isolated from 4-6 week old mice were cultured in methylcellulose (**a**) or liquid (**b**) media in the absence or presence of SB216763 as indicated. Colony numbers or cell counts were enumerated and expressed relative to untreated controls (\pm s.e.m.). Myeloid progenitors transduced by *MLL-AF6* were used for comparison. Representative colonies generated under the different inhibitor concentrations are shown beneath the respective bars (**a**).



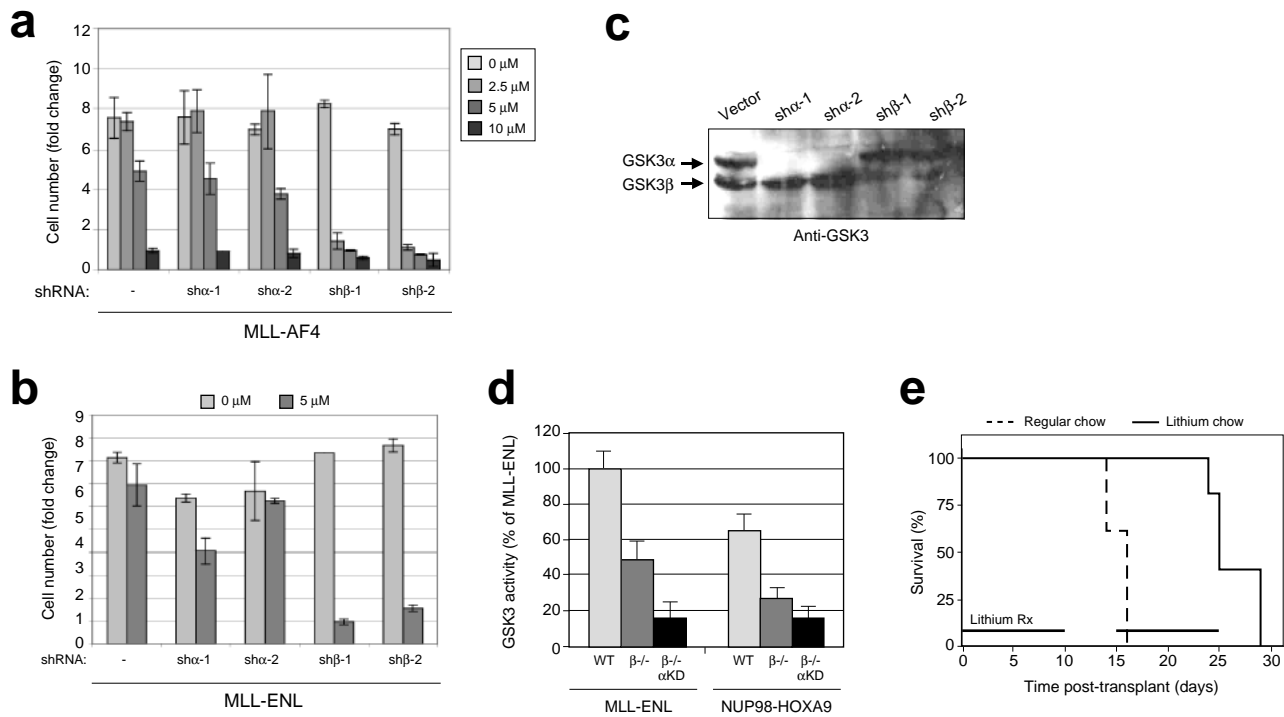
Supplementary Figure 5. Constitutively active AKT arrests the proliferation of *MLL* transformed cells.

a, Myeloid progenitors transformed by various oncogenes were transduced with constitutively active (CA-AKT) or kinase dead (KD-AKT) AKT constructs and then plated in methylcellulose medium containing cytokines. Colony numbers (\pm s.e.m., $n = 3$) were enumerated after 5 days incubation, and expressed relative to cell numbers obtained for cells transduced with empty vector. **b**, B cell progenitors transformed by *MLL-AF4* or the combination of *E2A-HLF* + *BCL-2* (BiEH) were transduced and analyzed as described in panel **a** (\pm s.e.m., $n = 3$). **c**, Transformed B cell progenitors were analyzed for GSK3 kinase activity in the absence (-) or presence (+) of 4-hydroxy-tamoxifen (4-OHT) to induce activation of conditional AKT. Results are expressed relative to uninduced cells (\pm s.e.m., $n = 3$). **d**, Transformed B cell progenitors transduced with ER-CA-AKT were analyzed by western blot using an anti-phospho GSK3 antibody, which shows rapid phosphorylation of GSK3 isoforms (upper panel) at specific times (indicated at top) following treatment with 4-OHT (0.1 mM). Lower panel shows total GSK3 isoform protein levels as detected with a non-phosphorylation sensitive antibody. **e**, Western blot analysis (anti-FLAG antibody) of transformed B cell progenitors demonstrates presence of FLAG-tagged exogenous wild type or S9A mutant GSK3 β . Lower panel shows total GSK3 isoform protein levels as detected with a pan-GSK3 antibody. **f**, Myeloid (upper panel) or lymphoid (lower panel) progenitors transformed by the indicated oncogenes and programmed to express conditional AKT (ER-CA-AKT) were transduced with exogenous wild type (WT) or mutant (S9A) GSK3 β or empty vector (Vector). After 3 days treatment with 4-OHT, cell numbers were determined and expressed relative to untreated cells (\pm s.e.m., $n = 3$).



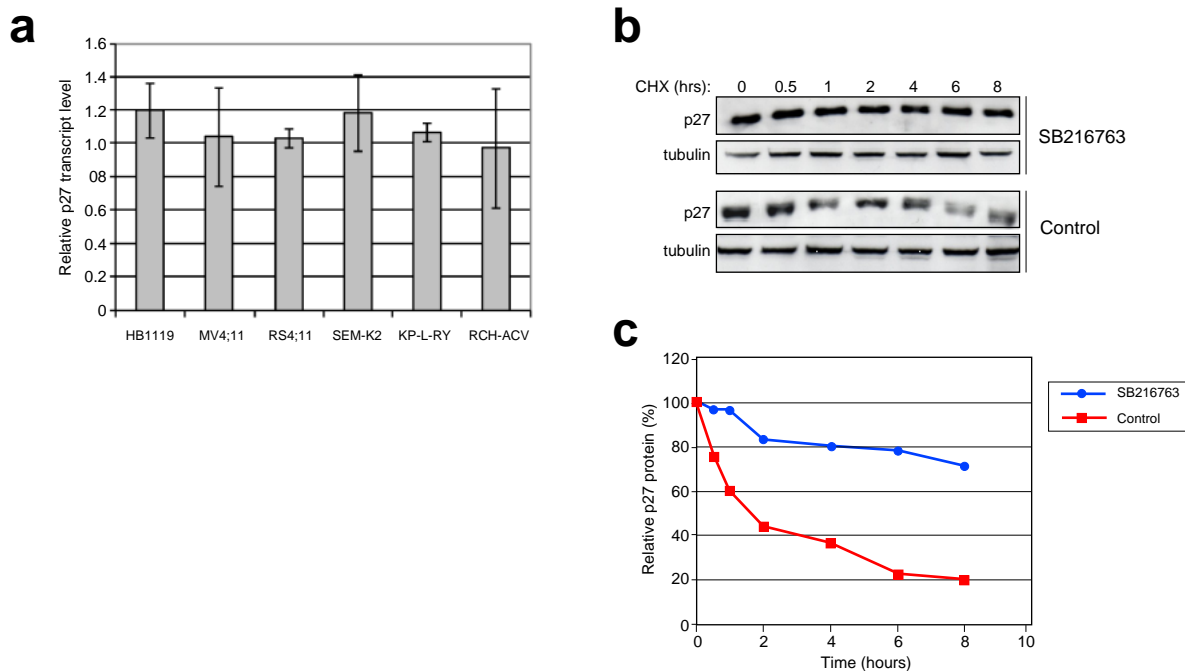
Supplementary Figure 6. Lack of *Gsk3b* does not abrogate the in vitro transformation of myeloid progenitors by various oncogenes.

Myeloid progenitors (c-kit⁺) enriched from *Gsk3b*^{-/-} fetal livers (E16) were transduced by the indicated oncogenes and assessed for their clonogenic potentials in serial replating assays as described in the METHODS. Colony numbers are shown through four rounds of plating for a representative experiment.



Supplementary Figure 7. *MLL*-transformed progenitors display increased sensitivity to GSK3 inhibition after knockdown of *Gsk3b* but not *Gsk3a*.

a, B cell progenitors immortalized by *MLL-AF4* and stably transduced with the indicated shRNA lentiviral vectors were incubated in the presence of different concentrations of SB216763. Cell numbers were determined after 3 days and expressed as fold increase relative to cell numbers at day 0 (\pm s.e.m., $n = 3$). **b**, Myeloid progenitors transformed by *MLL-ENL* and stably transduced with the indicated shRNA lentiviral vectors were incubated in the absence or presence of 5 μ M SB216763. Cell numbers were determined after 3 days and expressed as fold increase relative to cell numbers at day 0 (\pm s.e.m., $n = 3$). **c**, GSK3 protein levels were determined by western blot analysis of *MLL-ENL* cells following transduction with lentiviral vectors encoding shRNAs indicated above the respective gel lanes. Relative migrations of GSK3 α and GSK3 β isoforms are indicated on the left. **d**, Myeloid progenitors (WT, *Gsk3b* knockout and/or *Gsk3a* knockdown as indicated below the panel) transformed with the indicated oncogenes were assessed for GSK3 activity. Results are expressed relative to *MLL-ENL*-transformed wild type cells (\pm s.e.m., $n = 3$). **e**, Survival curves show significantly different latencies ($p < 0.001$) for development of acute leukaemia in cohorts of mice transplanted with *MLL-AF4* leukaemia cells (200,000) with *Gsk3b* knockdown. Mice were maintained on normal or lithium carbonate (0.4%) laced chow as indicated.



Supplementary Figure 8. GSK3 inhibition increases the half-life of p27 in *MLL*-transformed cells.

a, Human leukaemia cell lines (indicated below the panel) were cultured for 24 hours in the absence or presence of SB216763 (10 μ M) and then analyzed for p27 transcript levels by quantitative RT-PCR. Relative change in p27 transcript level (plus inhibitor/minus inhibitor) is shown (\pm s.e.m., $n = 3$). **b**, The human leukaemia cell line KP-L-RY was incubated in SB216763 (10 μ M) for 24 hrs, washed and then incubated with cycloheximide (50 μ g/ml) in the presence of inhibitor or vehicle (control). Cells removed at the indicated times (top) were analyzed by western blot for p27 or tubulin expression. **c**, Quantification of western blot data (average of two experiments) expressed as p27 level (corrected for respective tubulin level) relative to 0 time point.

Supplementary Table 1. Compounds employed for inhibition screen.

Compound	Target	Concentration range
Trichostatin A	Histone deacetylases	10-100 ng/ml
Histone deacetylase inhibitor II	Histone deacetylases	1-10 μ M
Histone deacetylase inhibitor III	Histone deacetylases	0.5-5 μ M
Olomoucine	CDKs	10-100 μ M
Iso-olomoucine	CDKs	10-100 μ M
Roscovitine	CDKs	5-50 μ M
GSK3 inhibitor IX (BIO)	GSK3/CDKs	0.3-3 μ M
PD98059	MEK	1-10 μ M
U0126	MEK	2-20 μ M
SB202190	p38 MAP kinase	1-10 μ M
SB203580	p38 MAP kinase	2-20 μ M
Proteasome inhibitor I	Proteasome	2.5-25 μ M
Lactacystin	Proteasome	2-20 μ M
MG-132	Proteasome	2.5-25 μ M
R0031-8220	Protein kinase C	1-10 μ M
Genistein	Protein tyrosine kinases	5-50 μ M
Jak3 inhibitor I	Jak3	10-200 μ M
PP2	Lck, FynT, Hck	0.1-20 μ M
AG490	Jak2	1-10 μ M
AG1296	PDGFR	1-10 μ M
AG1478	EGFR	1-20 μ M
KN-93	CaM kinase II	1-10 μ M
KT5720	Protein kinase A	0.5-5 μ M
Staurosporine	Ser/Thr kinases	0.03-0.3 μ M
Akt inhibitor	AKT	1-10 μ M
Rak kinase inhibitor I	Raf	0.5-5 μ M
Y-27632	ROCK	1-10 μ M
JNK inhibitor 1	Jun kinase	1-10 μ M
Rapamycin	mTOR	0.01 - 1 μ M
Wortmannin	PI-3 kinase	0.05-0.5 μ M

Supplementary Table 2. Human leukemia cell lines used for inhibitor screen.	
Cell line	Oncogene abnormality
RCH-ACV	E2A-PBX1
697	E2A-PBX1
KJ	E2A-PBX1
HAL-01	E2A-HLF
UOC-B1	E2A-HLF
YCH6	E2A-HLF
REH	TEL-AML1
NALM-6	TEL-PDGFRB
RS4;11	MLL-AF4
MV4;11	MLL-AF4
SEM-K2	MLL-AF4
KP-L-RY	MLL-AF5
K562	BCR-ABL

Supplementary Table 3. shRNA sequences		
Target transcript	shRNA	shRNA sequence
Gsk3b	shRNA1-forward	TGAAAGTTAGCAGAGATAAATTCAAGAGAT TTATCTCTGCTAACTTTCTTTTTTC
Gsk3b	shRNA1-reverse	TCGAGAAAAAAGAAAGTTAGCAGAGATAA ATCTCTTGAATTTATCTCTGCTAACTTTCA
Gsk3b	shRNA2-forward	TGAAAGTGATTGGAAATGGATTCAAGAGAT CCATTTCGAATCACTTTCTTTTTT
Gsk3b	shRNA2-reverse	TCGAGAAAAAAGAAAGTGATTGGAAATGG ATCTCTTGAATCCATTTCGAATCACTTTCA
Gsk3b	shRNA3-forward	TGGACCCAAATGTCAAATATTCAAGAGAT AGTTTGACATTTGGGTCTTTTTTC
Gsk3b	shRNA3-reverse	TCGAGAAAAAAGGACCCAAATGTCAAAT ATCTCTTGAATAGTTTGACATTTGGGTCCA
Gsk3a	shRNA1-forward	TGAAGTGGCTTACACTGACATTCAAGAGAT GTCAGTGTAAGCCACTTCTTTTTTC
Gsk3a	shRNA1-reverse	TCGAGAAAAAAGAAGTGGCTTACACTGACA TCTCTTGAATGTCAGTGTAAGCCACTTCA
Gsk3a	shRNA2-forward	TGAAGGTTCTTCAGGACAAATTCAAGAGAT TTGTCCTGAAGAACCTTCTTTTTTC
Gsk3a	shRNA2-reverse	TCGAGAAAAAAGAAGGTTCTTCAGGACAA ATCTCTTGAATTTGTCCTGAAGAACCTTCA
Gsk3a	shRNA3-forward	TGTACTACCGTGCTCCAGAATTCAAGAGAT TCTGGAGCACGGTAGTACTTTTTTC
Gsk3a	shRNA3-reverse	TCGAGAAAAAAGTACTACCGTGCTCCAGAA TCTCTTGAATTCTGGAGCACGGTAGTACA
p27	shRNA1-forward	TGAAGATGTCAAACGTGAGATTCAAGAGAT CTCACGTTTGACATCTTCTTTTTTC
p27	shRNA1-reverse	TCGAGAAAAAAGAAGATGTCAAACGTGAG ATCTCTTGAATCTCACGTTTGACATCTTCA
p27	shRNA2-forward	TGGTCAATCATGAAGAACTATTCAAGAGAT AGTTCTTCATGATTGACCTTTTTTC
p27	shRNA2-reverse	TCGAGAAAAAAGGTCAATCATGAAGAACT ATCTCTTGAATAGTTCTTCATGATTGACCA
p27	shRNA3-forward	TGCCAGGCGGTGCCTTTAATTTCAAGAGAA TTAAAGGCACCGCCTGGCTTTTTTC
p27	shRNA3-reverse	TCGAGAAAAAAGCCAGGCGGTGCCTTTAAT TCTCTTGAATTAAAGGCACCGCCTGGCA